Insulin stimulates glucose metabolism via the pentose phosphate pathway in *Drosophila* Kc cells

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Abstract ***Drosophila melanogaster*** **has become a prominent and convenient model for analysis of insulin action. However, to date very little is known regarding the effect of insulin on glucose uptake and metabolism in** ***Drosophila*****. Here we show that, in contrast to effects seen in mammals, insulin did not alter [^{3}H]2-deoxyglucose uptake and in fact decreased glycogen synthesis (~30%) in embryonic** ***Drosophila*** **Kc cells. Insulin significantly increased (~1.5-fold) the production of $^{14}CO_2$ from D-[1-^{14}C]glucose while the production of $^{14}CO_2$ from D-[6-^{14}C]glucose was not altered. Thus, insulin-stimulated glucose oxidation did not occur via increasing Krebs cycle activity but rather by stimulating the pentose phosphate pathway. Indeed, inhibition of the oxidative pentose phosphate pathway by 6-aminonicotinamide abolished the effect of insulin on $^{14}CO_2$ from D-[U-^{14}C]glucose. A corresponding increase in lactate production but no change in incorporation of D-[U-^{14}C]glucose into total lipids was observed in response to insulin. Glucose metabolism via the pentose phosphate pathway may provide an important source of 5′-phosphate for DNA synthesis and cell replication. This novel observation correlates well with the fact that control of growth and development is the major role of insulin-like peptides in** ***Drosophila*****. Thus, although intracellular signaling is well conserved, the metabolic effects of insulin are dramatically different between** ***Drosophila*** **and mammals.**

Key words: Insulin; *Drosophila*; Glucose metabolism; Cell growth

1. Introduction

The realization that insulin-stimulated intracellular signaling pathways are extremely well conserved from species as distantly related as humans and *Drosophila melanogaster* [1–3] has made the latter a prominent and convenient model for analysis of insulin action. Indeed, investigation of insulin signaling in *Drosophila* has produced many seminal discoveries in insulin signaling. The remarkable evolutionary conservation of intracellular insulin-signaling components includes insulin receptor substrates (IRS/*chico*), elements of the ras/mitogen-activated protein kinase (MAPK) pathway, phosphatidylinositol 3-kinase (PI3K), Akt/PKB and p70S6 kinase [4,5]. In *Drosophila*, mutations in any of these components result in reduction of the cellular and organismal growth; for example the *chico* mutant fly is only half the size of normal flies [6]. Such studies indicate that the insulin receptor signaling system is crucial to normal growth and development of *Drosophila* [2,6]. In addition, *chico* mutant flies present a two-fold increase in lipid levels when compared with their heterozygous siblings [6], suggesting that the insulin receptor signaling pathway also controls cellular metabolism in *Drosophila*. In mammals, the insulin receptor and its intracellular signaling cascades play a critical and well-characterized role in the regulation of glucose homeostasis [7,8].

An important step towards the understanding of glucose metabolism in *Drosophila* was the characterization of a glucose transport system in embryonic Kc cells [9] which could be inhibited by cytochalasin B and phloretin. Based on these data it was suggested that this insect cell line contained glucose transporters (Gluts) similar to those of mammalian cells, although Northern blot analysis using cDNA probes of rat Glut1, 2 and 4 failed to recognize any RNA species in *Drosophila* Kc cells [9]. More recently, the *Drosophila* Glut gene was cloned, showing highest homology with the human Glut1 and 4 [10]. The cDNA has an open reading frame encoding a protein of 480 amino acids which shows a similarity of 68% to the human Glut1 protein [10]. An almost identical degree of overall similarity with Glut4 was also reported; however, there were more gaps in the amino acid sequence alignment as compared to Glut1. In skeletal muscle and adipocytes of humans and rodents, insulin increases glucose uptake by translocating and activating Gluts, in particular Glut4. These occur via PI3K/PKB- and p38 MAPK-dependent signaling pathways, respectively [11,12]. In *Drosophila*, these insulin-signaling pathways are well conserved [3]. Thus, *Drosophila* appears to express the cellular machinery necessary to exhibit insulin-stimulated glucose transport. However, although the molecular mechanisms used by insulin to control carbohydrate metabolism have been extensively investigated in mammals [13], to date very little is known regarding the effect of insulin on glucose uptake and metabolism in *Drosophila*. Therefore, in this study we determined the relative importance of different pathways of glucose metabolism under basal and insulin-stimulated conditions in *Drosophila* Kc cells. We show that control of glucose metabolism in *Drosophila* differs markedly from that observed in mammals but correlates well with the growth-regulatory role of insulin in *Drosophila* [5].

2. Materials and methods

2.1. Glucose uptake

For all experiments *Drosophila* Kc cells were propagated at 26°C in Schneider's *Drosophila* media (Invitrogen, Canada) supplemented with

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10% fetal bovine serum, 50 units/ml penicillin, and 50 μg/ml streptomycin (all from Wisent, QC, Canada) and serum starved overnight prior to all experiments. To perform glucose uptake assays, Kc cells were harvested by centrifugation (2000 rpm, 3 min), and resuspended in buffer A (50 mM KH_2PO_4, 60 mM Na_2HPO_4, 50 mM NaCl, 100 mM KCl, 5 mM $MgSO_4$, 10 μM 2-deoxyglucose, pH 7.4) in Petri dishes. The uptake was monitored by adding [^{3}H]2-deoxyglucose (Amersham, QC, Canada, 1 μCi/ml) to the cell suspension which was maintained at room temperature either in the presence or absence of insulin (human insulin, Humulin®R, Eli Lilly, 1 μM). At specific time intervals (5, 10, 20, and 30 min), aliquots (200 μl) were withdrawn and layered over oil mixture (density 1.033 g/ml, by mixing 9 vol of silicone oil with 1 vol of mineral oil) in 1.5-ml microfuge tubes. The uptake was terminated by centrifugation through the oil at 13 000 × *g* for 30 s. The pellets were then solubilized with 0.1 ml of 1% sodium dodecyl sulfate in 75 mM NH_4HCO_3 and counted in scintillation fluid. Non-specific uptake was determined in the presence of cytochalasin B (10 μM) and was subtracted from all values.

2.2. Lactate production

Cells (10^7 per condition) were incubated with or without insulin for 2 h and the incubation medium (200 μl) was collected for lactate determination. Total lactate released in the medium was measured by the lactate oxidase assay using a kit from Sigma (St. Louis, MO, USA).

2.3. Glycogen synthesis

Glycogen synthesis was assessed by the incorporation of D-[U-^{14}C]glucose into glycogen as previously described [14]. Briefly, cells (10^7 per condition) were incubated for 2 h in serum-free medium containing 0.2 μCi/ml of D-[U-^{14}C]glucose in the presence or absence of insulin (1 μM). In order to test the role of PI3K in glycogen synthesis we also incubated the cells where indicated with wortmannin (100 nM) 30 min before the addition of insulin. Wortmannin was also present throughout the 2-h incubation period with or without insulin. Cells were harvested by centrifugation (2000 rpm, 3 min) and washed twice with ice-cold phosphate-buffered saline (PBS). Afterwards, 0.5 ml of KOH (1 M) was added to the pellet of cells obtained from each condition. Cell lysates were used for overnight glycogen precipitation with ethanol. Precipitated glycogen was dissolved in water and transferred to scintillation vials for radioactivity counting.

2.4. Production of $^{14}CO_2$ from D-[U-^{14}C]glucose

The production of $^{14}CO_2$ from D-[U-^{14}C]glucose was determined as previously described [15] with a few modifications. Briefly, cells (10^7/flask) were incubated for 2 h in starve medium containing 0.2 μCi/ml of D-[U-^{14}C]glucose in the presence or absence of insulin (1 μM), wortmannin (100 nM) or insulin+wortmannin. Each incubation plate had a centered well containing a piece of Whatman paper wet with 150 μl of phenylethylamine/methanol (1:1) to trap CO_2 produced during the incubation period. The plates were carefully sealed and after 2 h of incubation, 200 μl of H_2SO_4 (4 M) was added to the cells and incubated for an additional 1 h at room temperature. Finally, the pieces of Whatman paper were removed and transferred to scintillation vials for radioactivity counting. D-[U-^{14}C]glucose, D-[1-^{14}C]glucose and D-[6-^{14}C]glucose were from Amersham (QC, Canada). The contribution of the oxidative pentose phosphate pathway to total glucose oxidation was estimated from the specific yields of $^{14}CO_2$ from D-[1-^{14}C]glucose and D-[6-^{14}C]glucose as has been previously explained in detail [16,17]. The experimental procedures were similar to those described above for the determination of $^{14}CO_2$ production from D-[U-^{14}C]glucose. The inhibition of the oxidative pentose phosphate pathway was performed by adding 6-aminonicotinamide (1 mM) to the cells during the overnight starvation period [18]. 6-Aminonicotinamide was also present in the medium throughout the incubations with or without insulin.

2.5. Incorporation of D-[U-^{14}C]glucose into total lipids

The incorporation of D-[U-^{14}C]glucose into lipids was determined as previously described [19]. Briefly, 10^7 cells were incubated for 2 h with serum-free medium containing 0.2 μCi/ml of D-[U-^{14}C]glucose in the presence or absence of insulin (1 μM). Subsequently, the cells were centrifuged, medium discarded and the pellet of cells was washed three times with cold PBS and treated with 5 ml of Dole's reagent (isopropanol/*n*-heptane/H_2SO_4, 4:1:0.25) and radioactivity associated with the total lipid fraction extracted was determined by scintillation counting.

2.6. Statistical analysis

Data are presented as means ± S.E.M. Statistical analysis was performed by one-way analysis of variance (ANOVA) with Tukey–Kramer multiple comparison test or *t*-test (Instat Program). The level of significance was set at $P < 0.05$.

3. Results and discussion

Here we demonstrated a linear increase in glucose uptake in Kc cells over a 30-min period. This process was not altered by insulin (Fig. 1). Subsequent to glucose uptake, the ability of insulin to increase the rate of glycogen synthesis in mammalian tissues such as skeletal muscle is well characterized [8]. The mechanism by which this occurs involves the activation of PI3K and phosphorylation of PKB by phosphatidylinositol-dependent protein kinases (PDK-1 and PDK-2) with subsequent regulation of glycogen synthase kinase-3 (GSK-3) [20]. Although it has been reported that *Drosophila* GSK-3 [3,20] and glycogen phosphorylase [21], key enzymes in the regulation of glycogen synthesis and degradation, respectively, are closely related to the mammalian isoforms, to the best of our knowledge no studies have been carried out to establish if insulin exerts any regulatory effects on these key enzymes in *Drosophila*. Here, we measured the effect of insulin on glycogen synthesis in Kc cells. Surprisingly, in *Drosophila* Kc cells the incorporation of D-[U-^{14}C]glucose into glycogen was significantly reduced (~30%) in the presence of insulin (Fig. 2). This is opposite to what has been reported in mammals [8]. However, in agreement with our results, the insulin-related peptide of insects, bombyxin, has been reported to reduce the major storage carbohydrates in the silkworm *Bombyx mori* [22]. Furthermore, insulin-stimulated glycogen synthesis in skeletal muscle requires PI3K activation [23] yet the ability of insulin to decrease glycogen synthesis in Kc cells was not altered by preincubation with wortmannin (Fig. 2). This could be explained by the observation that GSK-3 in *Drosophila* can be regulated by an alternative pathway involving PKC rather than PI3K [24].

The production of $^{14}CO_2$ from D-[U-^{14}C]glucose was significantly increased (~1.5-fold) by insulin in Kc cells (Fig. 3A). This led us to suggest that insulin is shifting glucose metabolism towards oxidation in *Drosophila* Kc cells. However, the production of $^{14}CO_2$ from D-[U-^{14}C]glucose does not allow us to discriminate between the different pathways by which glucose is being oxidized in these cells. We have also measured $^{14}CO_2$ from D-[1-^{14}C]glucose and D-[6-^{14}C]glucose to evaluate the contribution of the oxidative PPP and of the Krebs cycle to oxidation, respectively. The average basal values for $^{14}CO_2$

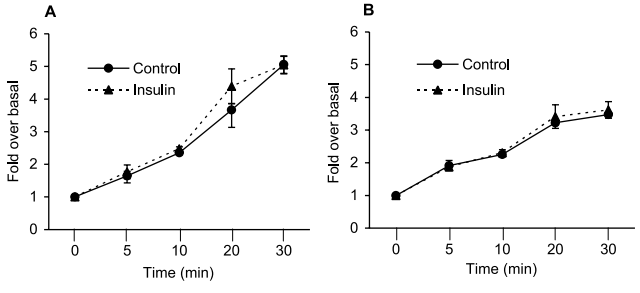

Fig. 1. Time course (0–30 min) of 2-deoxyglucose uptake in *Drosophila* (A) Kc and (B) S2 cells in the absence (control) or presence of insulin (1 μM). Data are presented as means ± S.E.M. from three independent experiments with triplicates.

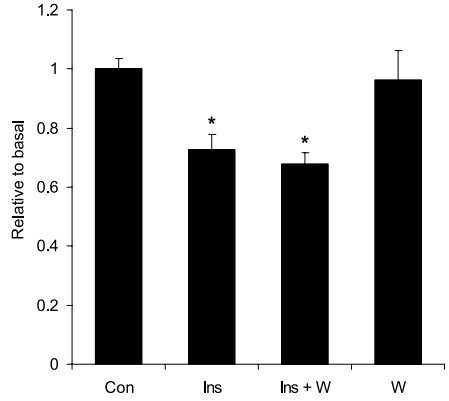

Fig. 2. Effect of insulin (Ins) on the incorporation of D-[U-^{14}C]glucose into glycogen. Cells were preincubated either in the absence (Con) or presence of wortmannin (W, 100 nM) for 30 min before the addition of Ins (1 μM). *$P < 0.05$ vs. Con and W (one-way ANOVA). Data are presented as means ± S.E.M. of four independent experiments with quadruplicates.

from D-[1-^{14}C]glucose were approximately 30 times higher than the values obtained from D-[6-^{14}C]glucose (~13.0 μM/10^7 cells vs. ~0.42 μM/10^7 cells, respectively). Interestingly, insulin significantly increased (~1.5-fold) the production of $^{14}CO_2$ from D-[1-^{14}C]glucose (Fig. 3B) while the production of $^{14}CO_2$ from D-[6-^{14}C]glucose was not altered by this hormone (Fig. 3C). This demonstrated that the activity of the Krebs cycle is very low and that the oxidative PPP accounts for almost all the production of $^{14}CO_2$ from D-[U-^{14}C]glucose. In fact, the oxidation rate from the PPP was ~30 times higher than from Krebs cycle. Therefore, we decided to use 6-aminonicotinamide, an inhibitor of the oxidative PPP, to confirm that insulin was in fact activating this pathway. The inhibition of the oxidative PPP by 6-aminonicotinamide abolished the insulin-stimulated effect of insulin on $^{14}CO_2$ from D-[U-^{14}C]glucose (Fig. 3D), confirming the oxidative PPP as the main metabolic pathway by which insulin increases glucose oxidation in *Drosophila* Kc cells. The activity of the PPP varies between different insects ranging anywhere from a contribution of only 2% to whole body glucose metabolism up to 80% of the total glucose metabolism in the fat body of silkworms [25]. Thus, our observation that the PPP is the main pathway by which *Drosophila* Kc cells oxidize glucose is in agreement with published data obtained from other species.

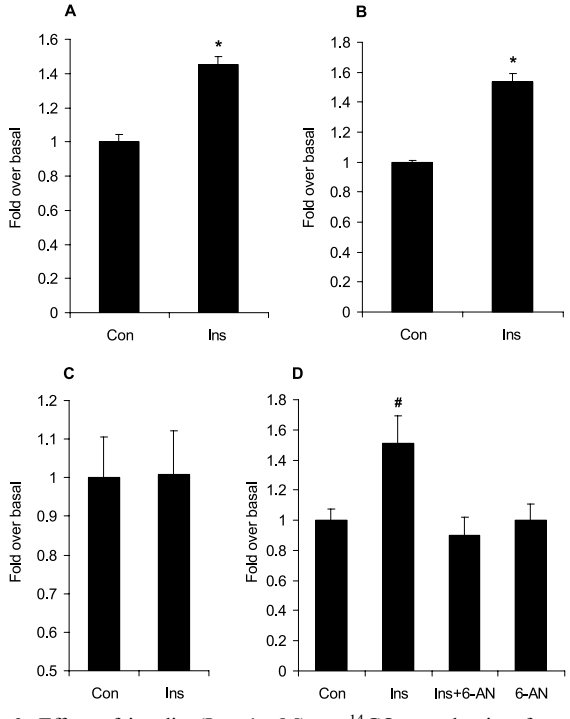

Fig. 3. Effect of insulin (Ins, 1 μM) on $^{14}CO_2$ production from (A) D-[U-^{14}C]glucose, (B) D-[1-^{14}C]glucose, (C) D-[6-^{14}C]glucose, and (D) D-[U-^{14}C]glucose in cells treated with the PPP inhibitor 6-aminonicotinamide (6-AN, 1 mM). *$P < 0.05$ vs. Con (*t*-test). #$P < 0.05$ vs. Con, Ins+6-AN and 6-AN (one-way ANOVA). Data are presented as means ± S.E.M. of three independent experiments with quadruplicates.

The PPP acts as an important source of biosynthetic precursors. In the first portion, which is oxidative, it fulfills two important cell requirements: (1) 5′-phosphates for the synthesis of nucleotides and nucleic acids, and (2) reducing power in the form of NADPH [26]. Additionally, in the second non-oxidative portion of the PPP, glyceraldehyde-3-phosphate and fructose-6-phosphate are produced [17]. These are intermediaries of the glycolytic pathway and may be channeled either to lactate production or oxidation in the Krebs cycle. However, in the Kc cells we found a very low activity of the Krebs cycle, therefore lactate production may be the most likely fate of these glycolytic intermediaries. Interestingly, it has been reported that flight muscle and fat body mitochondria are impermeable to either the oxidized or reduced forms of NAD in insects [25]. Therefore, energy generation to the cell depends to a large extent on the activity of the Embden–Myerhof glycolytic pathway in insects. In view of the presence of NAD in limited quantity in the cytosol and its importance as a cofactor in the oxidation of glyceraldehyde-3-phosphate in the glycolytic pathway, a mechanism for its reoxidation must be available in the cytosol in order for glycolysis to proceed. The formation of lactate by the reduction of pyruvate is an alternative to NAD reoxidation in the cytoplasm of insect cells [25]. In fact, we detected a significant increase (1.3-fold) in lactate production by the cells when they were exposed to insulin (Fig. 4). This is compatible with the metabolic characteristics of insect cells and also with the metabolic changes caused by insulin in Kc cells.

In mammalian cells, insulin promotes the synthesis of lipids, and inhibits their degradation [13]. Interestingly, in *Drosophila*, disruption of insulin signaling in *chico* mutant flies causes

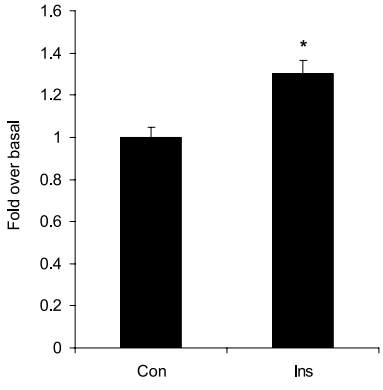

Fig. 4. Effect of insulin (Ins, 1 μM) on lactate production. *$P < 0.05$ vs. Con (*t*-test). Data are presented as means ± S.E.M. of three independent experiments with quadruplicates.

dyslipidemia. In fact, despite their smaller size, *chico* mutants have almost twice as much lipids as wild-type males per mg of fresh tissue, suggesting that insulin also regulates lipid metabolism in insects [6]. Here, we show that insulin did not affect lipid synthesis, measured as the incorporation of D-[U-^{14}C]glucose into total lipids (7.75 ± 0.68 and 8.03 ± 0.81 μmol/10^7 cells for control and insulin groups, respectively). The lack of an effect of insulin on D-[6-^{14}C]glucose decarboxylation and the low activity of the Krebs cycle either under basal or insulin-stimulated conditions may limit de novo lipid synthesis in Kc cells. However, this does not rule out the possibility that lipids may derive from other sources and their uptake and metabolism be differently regulated by insulin in *Drosophila*.

In summary, although the insulin-signaling machinery is clearly well conserved in *D. melanogaster* [3], the role of insulin in the direct control of glucose metabolism and energy storage in *Drosophila* is very different from that observed in mammals. Importantly, the effects of insulin on glucose metabolism reported by us here actually correlate with the well-documented growth-regulatory role of insulin in *Drosophila* [5]. The novel observation that insulin increased activity of the PPP may provide an important source of 5-phosphates for DNA synthesis in the control of *Drosophila* growth and development.

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